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16. ABSTRACT

A fathead minnow life-cycle exposure to various zinc concentrations demonstrated that the most sensitive indicators of zinc toxicity were egg adhesiveness and fragility, which were significantly affected at 145 µg Zn l^{-1} and above, but were not affected at 78 µg Zn l^{-1} and below. These effects occurred shortly after the eggs were spawned (during water hardening) and therefore were not related to effects on the parental fish. Hatchability and survival of larvae were significantly reduced, and deformities at hatching were significantly increased at 295 µg Zn l^{-1} and above. Acclimated and unacclimated groups of larvae exposed to identical zinc concentrations for 8 weeks after hatch showed only slight differences in senstivity.

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Toxic effects of zinc on fathead minnows *Pimephales promelas* in soft water

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A fathead minnow life-cycle exposure to various zinc concentrations demonstrated that the most sensitive indicators of zinc toxicity were egg adhesiveness and fragility, which were significantly affected at 145 μg Zn l^{-1} and above, but were not affected at 78 μg Zn l^{-1} and below. These effects occurred shortly after the eggs were spawned (during water hardening) and therefore were not related to effects on the parental fish. Hatchability and survival of larvae were significantly reduced, and deformities at hatching were significantly increased at 295 μg Zn l^{-1} and above. Acclimated and unacclimated groups of larvae exposed to identical zinc concentrations for 8 weeks after hatch showed only slight differences in sensitivity.

I. INTRODUCTION

Zinc is a common metal found in many effluent discharges (McKee & Wolf, 1963). Factors influencing the toxicity of zinc compounds and their toxic mode of action on fish and other aquatic animals have been reviewed and discussed by Skidmore (1964). Most research concerning zinc toxicity deals with short-term lethal exposures. Some information does exist, however, on long term sublethal effects of zinc on survival, growth, and reproduction of fish. A life-cycle exposure of the fathead minnow, *Pimephales promelas* Rafinesque to zinc in hard water was reported by Brungs (1969). He showed that the most sensitive indicator of zinc toxicity was a significant reduction in egg production at 180 μg Zn l^{-1}. In another closely related study Eaton (1973) conducted a life cycle exposure of fathead minnows to a mixture of copper, cadmium, and zinc in hard water. He also observed a significant reduction in egg production, which was attributed to the effects of zinc at 42 μg Zn^{-1}. Both authors, therefore, concluded that sublethal concentrations of zinc affected adult maturation, which directly affected the number of eggs spawned. Chronic tests with zinc have also been reported for flagfish *Jordanella floridae*, and rainbow trout, *Salmo gairdneri* (Sinley *et al.*, 1974; Spehar, 1976).

The present life cycle study was designed to establish a maximum acceptable toxicant concentration (MATC) for fathead minnows exposed to zinc in soft water. The results of this test could then be compared with the earlier exposures conducted in hard water, and the application factors calculated from all studies could be evaluated with regard to their use in environmental problems (Andrew *et al.*, in press).

In addition, a further evaluation of zinc effects on the early life stages was planned to determine the relationship between poor reproduction and egg adhesiveness and fragility (Brungs, 1969; Eaton, 1973), as well as the effects of acclimation on zinc toxicity during these early stages of development.

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II. METHODS

PHYSICAL AND CHEMICAL

Exposure system

A proportional diluter (Mount & Brungs, 1967) delivered 2 l of water per cycle to flow-splitting chambers (Benoit & Puglisi, 1973), which divided each of six concentrations four ways into dupilcate spawning tanks and duplicate larval growth tanks. Flow rates averaged 625 ml min^{-1} for spawning tanks and 95 ml min^{-1} for larval growth tanks. Replacement time for 90% of the test water was calculated to be approximately 4·5 h in spawning and larval growth tanks (Sprague, 1969). Spawning tanks (glass covered) were of stainless steel, 91 × 31 × 38 cm with a 31 cm standpipe. Larval growth tanks were located directly above and drained into the spawning tanks containing test solutions of the same concentration. They consisted of an outer glass tank, 55 × 28 × 15 cm and a removable inside tank, 43 × 26 × 15 cm, divided lengthwise into two 13-cm-wide larval growth chambers. Test water, which was divided evenly from the diluter and delivered to each duplicate growth tank, was again split through notches cut in the edge of each growth chamber. Equal amounts then flowed through both chambers, thereby forming four replicate larval growth chambers for each concentration. Water depth in each chamber measured 13 cm. All tanks were siphoned Monday to Friday and were scrubbed once a week. Egg cup screen bottoms were also cleaned daily to increase circulation of water around the embryos. Spawning tanks and growth chambers were concealed by curtains to minimize outside disturbance. Sylvania Gro-Lux and Duro-Test Vita-Lite*,† fluorescent lamps were used as the source of illumination, and the Evansville, Indiana, photoperiod was followed as recommended by the Committee on Aquatic Bioassays of the U.S. Environmental Protection Agency. Light intensity at the water surface of the spawning tanks ranged from 10 to 28 lumens.

Water characteristics

Water obtained directly from Lake Superior was passed through an ultraviolet sterilizer and then heated to a test temperature of 25 ± 1° C. Total hardness, alkalinity, acidity, and pH were determined on water from all tanks, alternately, one tank a week; dissolved oxygen was measured in a different duplicate tank twice a week. The means and ranges for total hardness, alkalinity, and acidity determinations were 46 (44–47), 41 (40–42), and 2 (1–6) mg l^{-1} as $CaCO_3$, respectively. The pH ranged from 7 to 8, and the mean for dissolved oxygen was 8 mg l^{-1} (range 6–9). Chemical measurements were made according to the American Public Health Association *et al.* (1971).

Toxicant solution

Zinc sulphate (granular $ZnSO_4$–$7H_2O$, regeant grade)‡ was dissolved in distilled water and introduced into the diluter by a metering device (Mount & Warner, 1965). Zinc concentrations were assigned to each test tank by stratified random assignment.

Water samples (25 ml per day) were taken Monday to Friday from each spawning and larval growth tank, acidified (0·2% HNO_3), and composited for 2 week periods. Zinc analyses of test water samples (Table I) were done by flame atomic absorption spectrophotometry (Perkin Elmer, 1976). One spiked water sample was run with each set of six samples, and three reference metal samples (Environmental Monitoring and Support Laboratory, Cincinnati, Ohio) of different zinc concentrations were run. Percentage recovery of 101 spiked samples was 102 ± 2·5%. The analyzed values of 52 sets of reference metal samples were 10 ± 2·6, 78 ± 4·6, and 371 ± 9·8 µg Zn l^{-1} compared to the specified values of 10, 79, and 367 µg Zn l^{-1}.

*The U.S. Environmental Protection Agency neither recommends nor endorses any commercial product; trade names are used only for identification.

†Sylvania Lighting Center, Danvers, Mass. 09123; and Duro-Test Corporation, Secaucus, N.J. 07094.

‡Mallinckrodt, Inc., St. Louis, Mo.

TABLE I. Survival and reproduction of fathead minnows exposed as eggs (<24 h after spawning) to various zinc concentrations, and survival of their offspring

Item	Total zinc (μg l^{-1})											
											Control	
Duplicate exposure tanks	577* ± 7 A	574 ± 8 B	295 ± 7 A	294 ± 7 B	152 ± 4 A	138 ± 4 B	78 ± 3 A	78 ± 3 B	46 ± 2 A	44 ± 2 B	2 ± 0·6 A	2 ± 0·9 B
Hatchability of eggs (%)†	77	83	92	97	79	92	93	92	83	93	89	83
8-week survival of fish (%)‡	0	0	64	28	100	92	96	100	100	72	96	88
Total spawnings found on substrates §			3‖	4‖	12‖	5	43	42	48	29	28	37
Total eggs adhering to spawning substrates¶			269‖	1138‖	2812‖	723‖	8461	5923	9299	5335	7604	9259
Chorions ruptured during removal from substrate (%)			66‖	47‖	41‖	31‖	9	14	2	2	4	4
Hatchability of offspring (%)			64‖	62‖	95	99	96	98	97	98	96	94
Deformed offspring at hatch (%)			11‖	40‖	17	17	4	4	1	4	10	1
8-week survival of offspring (%)**			18‖	18‖	96	86	98	100	98	100	100	98

*Mean and 95% confidence interval.
†Seventy-five eggs exposed per duplicate.
‡Twenty-five fish exposed per duplicate.
§Each of four spawning chambers per duplicate contained one male and one female.
‖Significantly different from control, $P=0{\cdot}05$ (analysis of variance with t test).
¶Eggs include those ruptured during removal from spawning substrates.
**Each entry represents the mean of two groups of 25 fish.

BIOLOGICAL

Acclimation and life-cycle exposures

First-generation fish were obtained from the stock unit maintained at our laboratory. The test was begun by distributing, at random, duplicate groups of 25 larvae (< 24 h after hatch) and 75 eggs (< 24 h after spawning) to each concentration. Larvae were placed directly in growth chambers. After 8 weeks the survival of these fish was calculated. Total length and weight of each fish were determined directly, and the fish were then discarded. Eggs were incubated in oscillating 0·12 l glass jars with 40-mesh nylon screen bottoms designed after those of Mount (1968). Nonviable eggs were removed from the cups the following day and discarded. Thereafter dead embryos were removed daily until hatch was complete. Twenty-five larvae from each cup were placed in growth chambers. Eight-week survival was also calculated for these fish. Total lengths were measured photographically as described by McKim and Benoit (1971), and all fish were transferred to spawning tanks of the same concentration. Larvae up to 2 weeks old were fed equal amounts of live San Francisco bay brine shrimp (< 8 h old) (Smith, 1976) three times a day; 2- to 3-week-old fish were fed two times a day, and 3- to 4-week-old fish were fed live shrimp once a day. All fish were gradually switched from live food to frozen adult brine shrimp, which was fed three times a day beginning 2 weeks after hatch.

Sex was determined on all fish 24 weeks after hatching by using Flickinger's (1969) method. At this time a 4-mesh stainless steel screen divider was also placed in each spawning tank, which divided the tank into four spawning chambers measuring $15 \times 31 \times 31$ cm high. One stainless steel spawning substrate coated with sand (Benoit, 1977) was placed in each spawning chamber. Four pairs of adults from each duplicate concentration were then chosen randomly and assigned to individual spawning chambers. All surplus adults were weighed, measured, and discarded.

Substrates were checked for spawnings daily, and the eggs were rolled off the substrate with a gentle circular motion of the finger (Gast & Brungs, 1973). The eggs produced in each spawning were counted, and 50 eggs from the first five spawnings and every third spawning thereafter from each pair were transferred to incubation cups and hatched. Egg chorions ruptured during removal from the substrate were also counted. Viability was determined 24 h after spawning on each group, and dead eggs were removed daily until hatching was complete. On completion of the hatch, dead or deformed larvae were counted, and 25 normal larvae were randomly selected from the first two spawnings in each duplicate spawning tank and placed in the growth chambers. These fish were fed the same diet as the parental fish.

Shortly after spawning commenced, an egg trap (Benoit, 1977) was placed under one of the four spawning substrates in each duplicate spawning tank. Eggs that fell into traps were counted each day and incubated 24 h to determine viability. These eggs were not incubated to hatching unless the majority of the spawning was found in the trap.

Adult fish were killed one week after the last spawning. The pairs that did not spawn were dissected for positive sexual identification, and it was found that all pairs were sexed correctly. Second-generation fish were killed 8 weeks after hatching. Survival of these fish was calculated, and total lengths and weights were measured directly. Survival, growth, and spawning data were subjected to a one-way analysis of variance with an F test ($P = 0{\cdot}05$) and Student's t test ($P = 0{\cdot}05$) (Steel & Torrie, 1960).

Acute toxicity exposures

Duplicate 96-h acute toxicity tests were conducted at $25 \pm 1°$ C with 4-week-old fathead minnows from the same stock used in the chronic tests. Continuous flow tests with the proportional diluter and 25 fish per concentration were conducted according to methods described by the U.S. Environmental Protection Agency (1975).

Egg adhesiveness and fragility

Two weeks before the end of the 12 week spawning period, one pair of adult fathead minnows exposed to 295 μg Zn l^{-1} and producing abnormal eggs was transferred to a control spawning chamber to determine if the pair could spawn normally.

After adult fish in the life-cycle test were killed, six pairs of unexposed mature fathead minnows were placed into spawning chambers containing egg traps and exposed to 295 μg Zn

l^{-1} for 1 week. Six pairs were also transferred for 1 week into control spawning chambers containing egg traps.

Several groups of eggs produced by two unexposed pair of fathead minnows were left on the spawning substrate and transferred intact before and after water hardening to 295 μg Zn l^{-1}. Eggs were then exposed for either 4 ot 24 h before removing them from the spawning substrate. Adherence of eggs to the substrates was noted, and chorion strength (fragility) was estimated by counting chorions ruptured during removal.

III. RESULTS

ACCLIMATION, LIFE-CYCLE AND ACUTE TOXICITY EXPOSURES

The eight week survival of fathead minnows exposed to zinc as eggs was reduced at mean concentrations of 295 and 576 μg Zn l^{-1} (Table I). Survival of fish exposed as larvae was also reduced at 295 and 576 μg Zn l^{-1} (16% and 0%, respectively). All deaths in both groups took place within 2 weeks after hatching. Growth of both groups was not affected at any concentration except where survival was low; the remaining fish were larger probably because of increased availability of food and space.

The total numbers of spawnings and eggs adhering to the spawning substrates (including those ruptured during removal) were significantly ($P=0{\cdot}05$) reduced at mean concentrations of 145 and 295 μg Zn l^{-1}, and significant ($P=0{\cdot}05$) numbers of eggs were consistently ruptured during removal from these spawning substrates (Table 1). Hatchability and 8 week survival of offspring were significantly ($P=0{\cdot}05$) reduced and deformities at hatching were significantly increased at 295 μg Zn l^{-1} (Table I). Growth was not affected by any of the exposure concentrations.

A large percentage of the eggs spawned were nonadhesive, and many of the spawnings were found entirely in the egg traps at 145 and 295 μg Zn l^{-1} (Table II). Viability of these eggs (Table II) was similar to those adhering to substrates.

The mean 96-h LC50 of duplicate continuous flow acute tests for fathead minnows was calculated by graphical extrapolation on semilogarithmic paper to be 600 μg Zn l^{-1}.

EGG ADHESIVENESS AND FRAGILITY

The pair of adult fish transferred from 295 μg Zn l^{-1} to control water successfully spawned twice in the control with an average of 202 eggs per spawning. Egg adhesiveness to the substrate was normal, and only 4% of the chorions ruptured during

TABLE II. Fathead minnow spawnings and eggs that fell into egg traps under selected spawning substrates at various zinc concentrations

Item	Total zinc (μg l^{-1})									
Individual spawning chamber*	295		145		78		45		(Control) 2	
	A	B	A	B	A	B	A	B	A	B
Spawnings found with eggs adhering to substrate	1	3	9	4	16	14	7	4†	7	8
Spawnings found entirely in trap with no eggs on substrate	12	6	0	8	0	0	0	0	0	0
Percentage of eggs (substrate + trap) found in trap	87	23	11	30	2	8	5	8	8	3
Egg viability in trap (%)	84	87	95	64	87	95	97	56†	90	97

*Each chamber contained one male and one female.
†Female became diseased and died.

TABLE III. Fathead minnow eggs spawned in control water and transferred intact (attached to the substrate) before and after water hardening to water containing 295 μg Zn 1^{-1}

Item	Before water hardening				After water hardening*	
Eggs spawned on substrate and transferred intact	14	20	39	68	54	17
Hours incubated after transfer	4	4	4	24	4	24
Chorions ruptured during removal from substrate (%)	21†	30†	15†	13†	0‡	6‡

*Eggs transferred were approximately 25-35 min old. At 25° C eggs became water hardened approximately 20 min after they were spawned.
†Eggs adhered poorly to spawning substrate and fell off with the slightest touch.
‡Eggs adhered normally to spawning substrate.

removal. Before transfer of this spawning pair to the control water only one group of 27 eggs was found on the substrate. These eggs fell off at the slightest touch, and 48% of the chorions were ruptured during removal.

Three of the six pairs of unexposed adults transferred to 295 μg Zn 1^{-1} spawned during the 1 week exposure. All eggs were found in the egg traps, and most chorions ruptured at the slightest touch. These spawnings were quite small, averaging only 23 eggs each; possibly many of the eggs were either broken or eaten by the fish before landing in the trap. Five of the six pairs of unexposed fish in the control chambers spawned within 1 week with an average of 117 eggs per spawning. Egg adhesiveness was normal, and only 5% of the chorions ruptured during removal.

Both adhesiveness and chorion strength of eggs spawned in control water and transferred intact (attached to the substrate) to 295 μg Zn 1^{-1} before water hardening were greatly reduced after an exposure period of only 4 h (Table III). Adhesiveness and fragility were not affected, however, when eggs were water hardened before exposure to 295 μg Zn 1^{-1}. Similar results were obtained when the exposure period was extended to 24 h (Table III). Egg viability of both groups was not affected and ranged from 73% to 89%.

IV. DISCUSSION

The most sensitive indicators of sublethal zinc toxicity to the fathead minnow were egg adhesiveness and fragility. Many eggs at 145 and 295 μg Zn 1^{-1} fell off the substrates soon after they were spawned, and fragility tests demonstrated that chorion strength was also reduced. These effects in soft water were apparently identical to those found in hard water by Brungs (1969) and Eaton (1973), who mentioned that spawned eggs in zinc concentrations were 'loosely attached' and 'consistently flaccid'. Both authors concluded that this condition was due to the effects of zinc on the maturation of adult fathead minnows, which directly affected the number of eggs spawned. In the present test, however, reduced egg adhesiveness and increased

fragility were not related to exposure of parental fish, which apparently developed normally at 145 and 295 μg Zn l^{-1}. Fish exposed to 295 μg Zn l^{-1} and producing abnormal eggs produced normal eggs within a few days after they were transferred to untreated water. Conversely, mature fathead minnows from untreated water produced abnormal eggs within a few days after they were transferred to 295 μg Zn l^{-1}, and further investigations revealed that effects on egg adhesiveness and fragility occurred before water hardening. In similar studies with brook trout (*Salvelinus fontinalis*) (Holcombe *et al.*, 1978), chorion strength was greatly reduced at zinc concentrations that killed brook trout embryos and larvae but not the parental fish. Egg fragility, therefore, appears to be an excellent indicator of the long term chronic effects of zinc on fish. This may also be true for other toxicants. McKim (1977) evaluated 56 life-cycle fish toxicity tests with 34 organic and inorganic chemicals and found that the embryo-larval stages usually showed a greater sensitivity than other stages in the life cycle. These findings further support investigations currently underway at this laboratory to determine the effect of various toxicants on the early development of fish.

Both groups of acclimated and unacclimated fathead minnows died within 2 weeks after hatching at 576 μg Zn l^{-1}, and only a small difference in survival was observed between the two groups exposed to 295 μg Zn l^{-1} (i.e. mean survival at 8 weeks was 46% acclimated larvae and 16% for unacclimated larvae). Skidmore (1965) also demonstrated that zebrafish (*Brachydanio rerio*) exposed to zinc as eggs were only slightly more resistant than newly hatched larvae, and the threshold concentrations for both groups appeared to be the same. Holcombe *et al.* (1978) found only a slight difference in zinc sensitivity between brook trout exposed as eggs and those exposed at the swim-up stage. Studies conducted by Sinley *et al.* (1974) and Spehar (1976) have shown, however, that unacclimated rainbow trout and flagfish were three to four times more sensitive to zinc than acclimated fish that were incubated and hatched in the same concentrations.

The zinc application factor (MATC/96-h LC50) reported here for fathead minnows in soft water with a pH of 7–8 is between 0·13 and 0·24, based on a MATC between 78 145 μg Zn l^{-1} and a 96-h LC50 of 600 μg Zn l^{-1}. The calculated MATC range is based on the reduction of egg adhesiveness and fragility. A review and discussion of the usefulness of zinc and other application factors has recently been completed by Andrew *et al.* (1977).

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